A Virus-like Particle of the Hepatitis B Virus preS Antigen Elicits Robust Neutralizing Antibody and T Cell Responses in Mice

Xiaodan Cai [a,†], Weihao Zheng [a,†], Shaokun Pan [b], Shengyuan Zhang [c], Youhua Xie [b], Haitao Guo [d], Guoxin Wang [e,*], Zigang Li [a,*], and Ming Luo [f,g,*]

Abstract

The preS antigen of hepatitis B virus (HBV) corresponds to the N-terminal polypeptide in the large (L) antigen in addition to the small (S) antigen. The virus-like particle (VLP) of the S antigen is widely used as a vaccine to protect the population from HBV infection. The presence of the S antigen and its antibodies in patient blood has been used as markers to monitor hepatitis B. However, there is very limited knowledge about the preS antigen. We generated a preS VLP that is formed by a chimeric protein between preS and hemagglutinin (HA), and the matrix protein M1 of influenza virus. The HBV preS antigen is displayed on the surface of preS VLP. Asn112 and Ser98 of preS in VLP were found to be glycosylated and *O*-glycosylation of Ser98 has not been reported previously. The preS VLP shows a significantly higher immunogenicity than recombinant preS, eliciting robust anti-preS neutralizing antibodies. In addition, preS VLP is also capable of stimulating preS-specific $CD8^+$ and $CD4^+$ T cell responses in Balb/c mice and HBV transgenic mice. Furthermore, preS VLP immunization provided protection against hydrodynamic transfection of HBV DNA in mice. The data clearly suggest that this novel preS VLP could elicit robust immune responses to the HBV antigen, and can be potentially developed into prophylactic and therapeutic vaccines.

Keywords: Hepatitis B virus; preS; IFN-γ; Glycosylation; Prophylactic efficacy; Therapeutic potential.

This is the author's manuscript of the article published in final edited form as:

Cai, X., Zheng, W., Pan, S., Zhang, S., Xie, Y., Guo, H., … Luo, M. (2018). A virus-like particle of the hepatitis B virus preS antigen elicits robust neutralizing antibodies and T cell responses in mice. Antiviral Research, 149, 48–57. https://doi.org/10.1016/j.antiviral.2017.11.007

Abbreviations: HBV, hepatitis B virus; VLPs, virus-like particles; M1, matrix protein of influenza virus; HA, hemagglutinin of influenza virus; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot assay; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; LC, liver cirrhosis; HCC, hepatocellular carcinoma; CTL, cytotoxic T lymphocyte; FBS, fetal bovine serum; PBS, phosphate buffered saline; qPCR, quantitative polymerase chain reaction; ALT, alanine aminotransferase; HBIG, hepatitis B immunoglobulin; PNGase F, Peptide -N-Glycosidase F; H&E, hematoxylin and eosin; FACS, flow cytometry.

Funding: This work was supported in part by the Natural Science Foundation of China Grants 21372023 and 81572198; MOST 2015DFA31590, the Shenzhen Science and Technology Innovation Committee JSGG20140519105550503, JCYJ20150331100849958, JCYJ20150403101146313, JCYJ20160301111338144, JCYJ20160331115853521 and JSGG20160301095829250; the Shenzhen Peacock Program KQTD201103.

Conflict of interest: M.L. and X.C. are the inventors of the intellectual property. The other authors declare no conflict of interest with respect to this manuscript.

ARTICLE INFORMATION:

From the [a]School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen, Guangdong 518055, P. R. China; [b]Key Laboratory of Medical Molecular Virology (MOE & MOH), Institute of Biomedical Sciences, School of Basic Medical Sciences, Shanghai Medical College, Fudan University, Shanghai 200032, China; [c]Chinese Academy of Sciences Key Laboratory of Infection and Immunity, Institute of Biophysics, 15 Da Tun Road, Beijing 100101, China; [d]Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN 46202, USA, [e]Research Center of Plasmonic and Near-Infrared Science, Research Institute of Tsinghua University in Shenzhen, South Area

of Hi-Tech Park, Nanshan, Shenzhen 518057, P. R. China; [f]Department of Chemistry, [g]Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30302, USA.

[†]These two authors made equal contributions to this work.

*** Corresponding authors.**

Ming Luo, Ph.D.

Department of Chemistry, Georgia State University, Atlanta, GA 30302, USA.

E-mail: mluo@gsu.edu

Tel: +1-404-413-6608; fax: +1-404-413-5505; or

Zigang Li, Ph.D.

School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen, Guangdong, 518055, P. R. China.

E-mail: lizg@pkusz.edu.cn

Tel: +86-755-2603-3616; or

Guoxin Wang, Ph.D.

Research Center of Plasmonic and Near-Infrared Science, Research Institute of Tsinghua University in Shenzhen, South Area of Hi-Tech Park, Nanshan, Shenzhen, 518057, P. R. China

E-mail: wanggx@tsinghua-sz.org

Tel: +86-755-2601-5166

1. Introduction

Under the current immunization and antiviral programs, hepatitis B virus (HBV) infection remains a major global public health problem. Approximately 2 billion people have been infected worldwide during their lifetime, and more than 350 million are chronic carriers of the virus. HBV infection may cause acute and chronic hepatitis, which leads to liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Current HBV vaccines on the market are based on the virus-like particle (VLP) of the S antigen, and effectively prevent most people from acquiring HBV infection. However, almost 5-10% people vaccinated with the available vaccines fail to mount an adequate antibody response to offer protection. As an additional antigen on infectious HBV particles, preS could also provide B and T cell epitopes which may promote the humoral and cellular responses and enhance the seroprotection rate by overcoming non-responsiveness to the S antigen-only vaccines [(4)].

There are three envelope proteins in the HBV virion, S, M and L. The preS protein is part of the L protein, a 163 amino acid extension at the N-terminus of the S protein (genotype A). preS may be further divided as preS1 (a.a.1-108) and preS2 (a.a.109-163). In the M protein, only preS2 is present at the N-terminus in addition to the amino acids that are common among the S, M and L proteins. Two well-known functions are related to preS. First of all, preS includes the region that interacts with the specific host receptor [(2)]. Previous studies have mapped the interaction motif to be within the first 48 amino acids of preS [(3)]. The other important function of preS is that in the preS region, there are highly immunogenic sites as B and T cell epitopes. It has been reported that preS could induce humoral responses in mice which were nonresponsive to the S antigen, indicating that preS represents a potential antigen for novel HBV vaccine candidates [(4)]. Notably, humoral response may play a major role in preventing HBV spreading to uninfected cells. In addition, it is generally believed that a proper $CD4^+$ helper T cell response is a prerequisite for an adequate humoral response. Furthermore, T cell responses may help extend the longevity of humoral immunity. However, how these preS

epitopes are related to virus clearance is not very clear. It is widely accepted that the $CD8^+$ T cell response is primarily responsible for HBV clearance in both cytopathic and noncytopathic manner, and the HBV-specific $CD8^+$ T cells are able to clear HBV from infected hepatocytes by secretion of antiviral cytokines, such as IFN-γ, and TNF-α.

VLPs resemble authentic native viruses in structure and morphology, but are non-infectious, because they assemble without containing the genetic material [(1)]. Compared to individual proteins or peptides, VLPs significantly improve humoral responses by presenting conformational epitopes more similarly as the native virus. Owing to their highly repetitive surface, VLPs are capable of eliciting robust B cell responses in the absence of adjuvants by efficiently cross-linking specific receptors on B cells. Besides, VLPs could also induce potent cytotoxic T lymphocyte (CTL) responses in immunized animals.

In this study, we have designed a preS VLP that consists of influenza M1 protein and the transmembrane domain and cytoplasmic tail of influenza hemagglutinin (HA) as the scaffold. The HBV preS antigen is displayed on the surface of VLP when it is fused with the HA fragment. We assessed the immunogenicity of preS VLP in mice. Here we report that immunization with preS VLP induced both potent humoral and cellular immune responses in Balb/c mice and HBV transgenic mice, and protected mice from hydrodynamic transfection of HBV DNA, indicating its potential as an effective prophylactic vaccine candidate or a potential therapeutic vaccine against HBV infection.

2. Materials and methods

Materials and methods are provided in the Supporting Information.

3. Results

3.1 Construction and preparation of preS VLP

Co-expression of influenza virus M1 and HA releases a VLP decorated with HA antigen [11]. In order to generate a VLP that is decorated with HBV preS antigen, we constructed a chimeric protein that has the preS sequence fused at the N-terminus of a HA fragment that includes its transmembrane domain and the cytoplasmic tail (Fig. 1A). The signal peptide from HA was also added in front of the preS sequence which may be removed after the chimeric protein is expressed. The amino acid 41 of M1 was mutated to Ala to enhance the release of the VLP [12]. After transfection of 293T cells with pCAGGS-M1, pCAGGS-preS-HA, or both plasmids, respectively, total RNAs were extracted and analysed for the transcription levels of M1 and preS-HA. The qRT-PCR results suggested that both M1 and preS-HA genes had been transcribed adequately in 293T cells 48 h after transfection (Fig. 1B). After co-transfection of pCAGGS-M1 and pCAGGS-preS-HA into 293T cells, expression of the preS antigen was readily detected by immunofluorescent microscopy (Fig. 1C and 1D). If the M1 protein was not co-expressed, the preS-HA chimeric protein appeared to be unable to expose the preS antigen on the exterior of the cellular membrane because the preS antigen was only detectable after the cellular membrane was permeabilized with Triton X-100 (Fig. 1C and 1D).

There has been an interesting observation of L antigen topology [13-15]. It was found that the preS region of the L antigen may be at both the side of cytosol or the side of lumen of ER membrane [13]. The association of the preS region with the S region in the L antigen is critical to the preS topology of the L antigen [14]. In our construct of VLP, the S region of the L antigen is not present and the N-terminus of preS may not be myristoylated. preS in the chimeric protein is completely in the cytosol side when preS-HA is expressed alone. In co-expression, M1 allows preS to be presented on the exterior of the cytoplasmic membrane, likely through its interactions with the cytoplasmic tail of HA. In addition, M1 also plays the critical role of assembly and

budding of VLP (see below).

3.2 Purification and characterization of preS VLP

Next, we set out to purify the preS VLP from supernatant of the transfected 293T cells. Recombinant preS was also produced in *E. coli* [5] (Fig. S1) and was used to generate specific polyclonal antibodies. The culture media were collected from the cells 72 h post-cotransfection of pCAGGS-M1 and pCAGGS-preS-HA. The culture media were laid on a sucrose gradient and subjected to ultracentrifugation. A sample was collected from each of the 30%, 40% or 50% sucrose fractions. By SDS-PAGE and western blot analysis, the protein corresponding to the preS-HA antigen was identified (Fig. 2A and 2B). Based on western blot analysis, the secreted preS-HA spans a region of much larger molecular weight than the calculated molecular weight of the preS-HA chimeric protein. We concluded that the secreted forms of preS-HA are glycosylated, consistent with previous reports of the L antigen [16-18]. A band consistent with the molecular weight (27.8 kDa) of M1 was shown in Fig. 2A. This could account for the appearance of an unstained region (about 27.8 kDa) in western blot due to that M1 occupies the position (Fig. 2B and 2C). The sample was further characterized by liquid chromatograph-mass spectrometer/mass spectrometer (LC-MS/MS). The M1 sequence identified by MS analysis covered above 90% of the full length M1 sequence (Fig. S2). A negative stain electron micrograph showed that the sucrose fractions contain virus-like particles (Fig. 2E). These data showed that VLPs composed of M1 and preS-HA proteins were successfully made and purified.

To map glycosylation sites, we used Peptide -N-Glycosidase F (PNGase F) and/or *O*-Glycosidase to treat the 40% sucrose fraction, respectively. When preS VLP was treated with PNGase F to remove N-linked glycans, and O-Glycosidase to remove O-linked glycans, or both, a band with reduced molecular weight was observed (Fig. 2C). The molecular weight of the band is consistent with that of preS-HA. When O-Glycosidase was used alone, a smaller portion of deglycosylated preS-HA was observed. The remaining protein also seemed to have a lower

molecular weight, suggesting that the majority of preS-HA in VLP was modified with both N-linked and O-linked glycans. This is consistent with the pattern when both glycosidases were used to treat preS VLP. When PNGase F was used alone, on the other hand, a more significant amount of deglycosylated preS-HA was observed, suggesting a good portion of preS-HA in VLP contains only N-Linked glycans. preS VLP was further characterized by LC-MS/MS and analysed by Byonic. Both preS-HA and M1 sequences identified by MS analysis covered above 85% of the full length sequences (Fig. S2). Asn112 and Ser98 within the preS domain were predicted to be modified by N-glycan and O-glycan, respectively (Fig. 2D, and Table S1). N-glycosylation of Asn4 and Asn112 has been reported previously.[18] However, we did not identify peptides containing glycosylated Asn4, which may be due to the experimental condition of MS. Interestingly, *O*-glycosylation of Ser98 has not been reported previously, the physiological role of *O*-glycosylated Ser98 remains to be elucidated.

3.3 preS VLP elicits robust neutralizing antibodies in Balb/c mice

In order to investigate humoral immunogenicity of preS VLP, Balb/c mice were immunized each with 10 μg of preS VLP total protein, or recombinant preS [5] with alum adjuvant, respectively (Fig. 3A). PBS was used as a mock control. A booster was given on day 22. Blood samples were collected from each mouse on day 52 and 112, and sera were prepared from these samples. All serum samples were diluted by various folds before the serum antibody titers were determined by ELISA (Fig. S5). The data revealed that preS VLP is more potent than recombinant preS protein in generating anti-preS antibodies, even without the use of any adjuvant (Fig. 3B-D). In particular, preS VLP elicited high level anti-preS total IgG including both anti-preS IgG1 (Th2 isotype) and IgG2a (Th1 isotype), indicating a balanced Th1/Th2 response against preS VLP.

To further test whether anti-preS VLP sera could block HBV from infecting human hepatocytes, in vitro infection experiments were conducted. Using hepatitis B immunoglobulin

(HBIG) as a positive control, the anti-preS VLP sera from three mice clearly prevented HBV from infecting HepG2/hNTCP cells, as demonstrated by a decreased level of HBeAg in the supernatant of cell culture (Fig. 3E). Collectively, these results indicate that preS VLP can stimulate anti-preS neutralizing antibodies in mice.

3.4 preS VLP provokes strong T cell responses in Balb/c mice

T cell responses play a vital role in the induction of humoral immunity, and are crucial to effectiveness of a therapeutic HBV vaccine [19, 20]. To evaluate whether preS contained vital immunogenic epitopes to elicit strong T cell responses, we used preS VLP and preS protein to immunize Balb/c mice. Splenocytes were harvested and stimulated with preS-specific T cell peptides (Table 1) [21-23]. After stimulation for 6 h, T cell responses were determined using flow cytometric analysis (FACS) and ELISPOT (Fig. 4). FACS data showed that mice immunized with preS VLP elicited a higher percentage of preS-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells than either the controls or those immunized with recombinant preS protein (Fig. 4A, and 4B). ELISPOT also demonstrated that preS-specific IFN-γ-producing T cells are more abundant in mice immunized with preS VLP than either the controls or those immunized with recombinant preS protein (Fig. 4C and 4D). Since IFN-γ-producing T cells play a key role in controlling and clearing HBV in infected individuals, our results implicate that preS VLP can evoke potent preS-specific T cell responses that may be important in HBV clearance.

3.5 preS VLP provides protection against hydrodynamic transfection of HBV DNA.

To test whether preS VLP-induced T cell responses played a role in protection against HBV infection, mice immunized with preS VLP or preS protein were hydrodynamically transfected with HBV DNA on day 70 of post-immunization (Fig. 3A). HBV replication was induced by hydrodynamic injection of pHBV1.3 plasmid (10 μg/mouse) that contains a 1.3-fold-overlength genome of HBV. Liver tissues were collected on day 77 for analysis of HBV RNA

copies, viral antigen load, and humoral responses. HBV RNA copies from about 50 mg of each tissue sample were measured by qRT-PCR (Fig. 5A). The levels of HBV RNA in preS VLP-immunized animals were lower than that in animals immunized with recombinant preS. Liver sections stained for HBV core antigen indicated that the HBcAg-positive hepatocytes were eliminated almost entirely in preS VLP-immunized animals, but persisted in preS-immunized animals (Fig. 5B). We also used ELISA assays to detect HBV proteins including HBsAg and HBeAg in the serum samples collected from each immunized mouse on day 0, 2, 4 and 7 after hydrodynamic transfection of HBV DNA. As shown in Fig. 5C, HBsAg levels in preS VLP-immunized animals were nearly undetectable throughout 7 days, but rose to high levels in animals immunized with recombinant preS on day 4 after transfection. The serum levels of HBeAg also remained nearly undetectable in preS VLP-immunized animals over the course of 7 days. On the other hand, HBeAg levels in preS-immunized animals elevated on day 4 then dropped to nearly undetectable levels on day 7 after transfection (Fig. 5D). This phase of HBsAg and HBeAg clearance coincides with the development of anti-preS neutralizing antibodies (Fig. 5E). In line with previous study [7], serum alanine aminotransferase (ALT) activity increased significantly on day 2, indicative of successful transfection (Fig. 5F). Subsequently, the ALT activity of all mice returned to baseline by day 7 after transfection, implying that preS VLP did not cause liver damage. This was further supported by H&E staining, which suggested that no obvious necroinflammatory lesions were observed in liver section of mice from preS VLP group on day 7 after transfection (Fig. S3). Collectively, preS VLP prophylactically protects mice against hydrodynamic transfection of HBV DNA.

3.6 preS VLP immunization stimulates potent humoral and cellular responses in HBV transgenic mice

We next set out to investigate the therapeutic potential of preS VLP immunization by employing HBV transgenic mice as a model of chronic infection. HBV transgenic mice were first primed with preS VLP or PBS, and then were boosted on days 22 and 43, respectively. Despite that HBV transgenic mice have become tolerance to HBV [24], preS VLP could still induce high levels of anti-preS total IgG including both anti-preS IgG1 (Th2 isotype) and IgG2a (Th1 isotype) (Fig. 6B-D), suggesting a balanced Th1/Th2 response against preS VLP. In addition, preS VLP immunization could stimulate a higher percentage of preS-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells than the control by FACS analysis (Fig. 6E, and 6F). Furthermore, ELISPOT results also showed that preS-specific IFN-γ-producing T cells are more plentiful in mice immunized with preS VLP than the control (Fig. 6G and 6H), implying the therapeutic potential of preS VLP. Taken together, preS VLP vaccine could induce preS-specific $CD8^+$ and $CD4^+$ T cell responses in HBV transgenic mice.

4. Discussion

Previous studies of immune responses to the preS antigen, or preS1 and preS2, were largely carried out with recombinant proteins or synthetic peptides [26-32]. These polypeptides do not have the folded structure or glycosylation of preS found in HBV particles. The full extent of immune responses to the preS antigen may not have been recapitulated by these reports. We have successfully developed a unique system to produce preS VLP. In contrast to recombinant preS, preS VLP is able to evoke much higher levels of neutralizing antibodies in Balb/c mice, even without any adjuvants. This is likely attributed to that preS VLPs display highly repetitive, glycosylated and folded preS antigen on the surface. Importantly, the sera from mice immunized with preS VLP is capable of blocking HBV from infecting HepG2/hNTCP cells in vitro. Moreover, the preS VLP could provide protection against HBV in the hydrodynamic transfection model. Anti-preS1 antibodies were previously found to be effective in neutralization of HBV [33]. The identification of protective epitopes within the preS1 region reveals that preS1 specific

antibodies neutralize the virus by blocking the binding of host NTCP receptor [2, 29]. The potent antigenicity of preS VLP suggests that its preS antigen may have similar immunological properties of HBV particles.

Therapeutic vaccination requires multiple T cell responses, especially the $CD8^+$ T cell responses [19, 20]. It is generally believed that immunization with a therapeutic vaccine would stimulate the immune system to elicit a specific $CD8^+$ T cell response that is capable of controlling viral infection. In particular, therapeutic vaccination is fascinating for HBV because individuals who become chronically infected have a weak and narrowly focused $CD8^+$ T cell response [34, 35]. Compared to chronically infected individuals, there are greater numbers of HBV-specific IFN-γ secreting $CD4^+$ and $CD8^+$ T cells in individuals that resolve acute infections [36]. Indeed, preS VLP induce potent $CD4^+$ and $CD8^+$ T cell responses both in Balb/c mice and HBV transgenic mice. The T cell epitopes of preS used in this study clearly reactivated specific T cell responses. In addition, humoral immune responses could also be induced by preS VLP in HBV transgenic mice. These findings suggest that preS VLP may have the therapeutic potential for chronic HBV infection.

Interestingly, the preS domain of VLP was identified to be N-glycosylated at Asn112 and *O*-glycosylated at Ser98. *O*-glycosylation of Ser98 has not been reported previously. Alignment of the primary sequences of preS from all HBV genotypes revealed that this residue is highly conserved except that a threonine is at position 98 in genotype E HBV preS (Fig. S4). Notably, the N-glycosylation of preS is crucial for secretion and stability of preS antigen [37]. The physiological role of *O*-glycosylation of Ser98 and relationship between glycosylation and immunogenicity await further studies.

Due to the additional B and T cell epitopes in HBV preS region, preS represents an attractive antigen for HBV vaccine candidates that are able to overcome non-responsiveness to the S antigen-based vaccines, or break immune tolerance in patients with chronic HBV

infections [4, 38]. Previous clinical trials have shown that vaccines containing preS/S are effective and even superior to traditional S protein containing vaccine [39-42]. This may be due to the stimulation of preS-specific antibodies; however, the role of anti-preS antibodies was not evaluated in these studies. On the other hand, such epitopes may contribute to the better immunogenicity of these vaccines, although T cell immune responses induced by specific T cell epitopes from preS have not been defined.

The preS antigen has been exploited previously as candidates of prophylactic or therapeutic vaccines. When recombinant preS proteins were used in immunization, the antibody titers were not very high [43]. The ability of neutralizing HBV by these antibodies is limited. More critically, no T cell responses against HBV were demonstrated [44, 45]. This is consistent with that isolated proteins generally do not induce T cell responses because they usually are not internalized by antigen presenting cells [46]. In order to overcome the barrier, virus or yeast vectors were employed to express HBV antigens [47, 48]. However, there are safety concerns when a replicating vector is used despite their capability to induce strong T cell responses. In addition, these vectors may only be given once because the host will establish immunity against the vector. A recent study showed that administration of preS-based allergen-derived peptides with Alum adjuvant induced preS-specific antibodies that inhibit hepatitis B infection in vitro [27]. However, the stability of this peptide vaccine may be the limitation for clinical use. Furthermore, the preS in this peptide vaccine may not adopt a similar conformation as preS in HBV particles. Our design of preS VLP has the advantages of mimicking a virus particle and being able to interact with the antigen presenting cells to elicit both $CD4^+$ and $CD8^+$ T cell responses, and at the same time, it permits multiple doses and does not replicate any foreign microorganisms. In summary, preS VLP represents an effective vaccine candidate for prophylactic protection and potential therapeutic intervention against HBV infection.

Acknowledgements

We thank Professor John Steel of Emory University School of Medicine for providing the cDNAs of influenza virus used in this study. We thank Professor Andy (Qiqui) Yu Indiana University School of Medicine for discussions and comments.

References

1) Grgacic EV, Anderson DA. Virus-like particles: passport to immune recognition. Methods 2006;40:60-65.

2) Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife 2012;1:e00049.

3) Glebe D, Urban S, Knoop EV, Cag N, Krass P, Grun S, et al. Mapping of the hepatitis B virus attachment site by use of infection-inhibiting preS1 lipopeptides and tupaia hepatocytes. Gastroenterology 2005;129:234-245.

4) Milich DR. T- and B-cell recognition of hepatitis B viral antigens. Immunol Today 1988;9:380-386.

5) Lian M, Zhou X, Wei L, Qiu S, Zhou T, Li L, et al. Serum levels of preS antigen (HBpreSAg) in chronic hepatitis B virus infected patients. Virol J 2007;4:93.

6) Zheng W, Liang Y, Zhao H, Zhang J, Li Z. 5,5'-Methylenedisalicylic Acid (MDSA) Modulates SarA/MgrA Phosphorylation by Targeting Ser/Thr Phosphatase Stp1. Chembiochem 2015;16:1035-1040.

7) Yang PL, Althage A, Chung J, Chisari FV. Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. Proc Natl Acad Sci U S A 2002;99:13825-13830.

8) Wang W, Sun L, Li T, Ma Y, Li J, Liu Y, et al. A human monoclonal antibody against small envelope protein of hepatitis B virus with potent neutralization effect. MAbs 2016;8:468-477.

9) Ye X, Zhou M, He Y, Wan Y, Bai W, Tao S, et al. Efficient Inhibition of Hepatitis B Virus Infection by a preS1-binding Peptide. Sci Rep 2016;6:29391.

10) Zheng W, Cai X, Xie M, Liang Y, Wang T, Li Z. Structure-Based Identification of a Potent Inhibitor Targeting Stp1-Mediated Virulence Regulation in Staphylococcus aureus. Cell Chem Biol 2016;23:1002-1013.

11) Galarza JM, Latham T, Cupo A. Virus-like particle (VLP) vaccine conferred complete protection against a lethal influenza virus challenge. Viral Immunol 2005;18:244-251.

12) Campbell PJ, Kyriakis CS, Marshall N, Suppiah S, Seladi-Schulman J, Danzy S, et al. Residue 41 of the Eurasian avian-like swine influenza a virus matrix protein modulates virion filament length and efficiency of contact transmission. J Virol 2014;88:7569-7577.

13) Prange R, Streeck RE. Novel transmembrane topology of the hepatitis B virus envelope proteins. EMBO J 1995;14:247-256.

14) Lambert C, Mann S, Prange R. Assessment of determinants affecting the dual topology of hepadnaviral large envelope proteins. J Gen Virol 2004;85:1221-1225.

15) Bruss V. Hepatitis B virus morphogenesis. World J Gastroenterol 2007;13:65-73.

16) Hassemer M, Finkernagel M, Peiffer KH, Glebe D, Akhras S, Reuter A, et al. Comparative characterization of hepatitis B virus surface antigen derived from different hepatitis B virus genotypes. Virology 2017;502:1-12.

17) Schmitt S, Glebe D, Tolle TK, Lochnit G, Linder D, Geyer R, et al. Structure of pre-S2 N- and O-linked glycans in surface proteins from different genotypes of hepatitis B virus. J Gen Virol 2004;85:2045-2053.

18) Lambert C, Prange R. Posttranslational N-glycosylation of the hepatitis B virus large envelope protein. Virol J 2007;4:45.

19) Celis E, Kung PC, Chang TW. Hepatitis B virus-reactive human T lymphocyte clones: antigen specificity and helper function for antibody synthesis. J Immunol 1984;132:1511-1516.

20) Chisari FV, Isogawa M, Wieland SF. Pathogenesis of hepatitis B virus infection. Pathol Biol 2010;58:258-266.

21) Doh H, Roh S, Lee KW, Kim K. Response of primed human PBMC to synthetic peptides derived from hepatitis B virus envelope proteins: a search for promiscuous epitopes. FEMS Immunol Med Microbiol 2003;35:77-85.

22) Pajot A, Michel ML, Mancini-Bourgine M, Ungeheuer MN, Ojcius DM, Deng Q, et al. Identification of novel HLA-DR1-restricted epitopes from the hepatitis B virus envelope protein in mice expressing HLA-DR1 and vaccinated human subjects. Microbes Infect 2006;8:2783-2790.

23) Ferrari C, Cavalli A, Penna A, Valli A, Bertoletti A, Pedretti G, et al. Fine specificity of the human T-cell response to the hepatitis B virus preS1 antigen. Gastroenterology 1992;103:255-263.

24) Allweiss L, Dandri M. Experimental in vitro and in vivo models for the study of human hepatitis B virus infection. J Hepatol 2016;64:S17-31.

25) Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity 1996;4:25-36.

26) Lempp FA, Urban S. Inhibitors of hepatitis B virus attachment and entry. Intervirology 2014;57:151-157.

27) Cornelius C, Schoneweis K, Georgi F, Weber M, Niederberger V, Zieglmayer P, et al. Immunotherapy With the PreS-based Grass Pollen Allergy Vaccine BM32 Induces Antibody Responses Protecting Against Hepatitis B Infection. EBioMedicine 2016;11:58-67.

28) Chen Y, Bai Y, Guo X, Wang W, Zheng Q, Wang F, et al. Selection of affinity-improved neutralizing human scFv against HBV PreS1 from CDR3 VH/VL mutant library. Biologicals 2016;44:271-275.

29) Sankhyan A, Sharma C, Dutta D, Sharma T, Chosdol K, Wakita T, et al. Inhibition of preS1-hepatocyte interaction by an array of recombinant human antibodies from naturally recovered individuals. Sci Rep 2016;6:21240.

30) Kim JH, Gripon P, Bouezzedine F, Jeong MS, Chi SW, Ryu SE, et al. Enhanced humanization and affinity maturation of neutralizing anti-hepatitis B virus preS1 antibody based on antigen-antibody complex structure. FEBS Lett 2015;589:193-200.

31) Zhang Z, Li X, Yi W, Li S, Hu C, Chen A. A monoclonal antibody specific to the non-epitope region of hepatitis B virus preS1 contributes to more effective HBV detection. Clin Biochem 2013;46:1105-1110.

32) Toita R, Kawano T, Kang JH, Murata M. Applications of human hepatitis B virus preS domain in bio- and nanotechnology. World J Gastroenterol 2015;21:7400-7411.

33) Neurath AR, Seto B, Strick N. Antibodies to synthetic peptides from the preS1 region of the hepatitis B virus (HBV) envelope (env) protein are virus-neutralizing and protective. Vaccine 1989;7:234-236.

34) Barnaba V, Franco A, Alberti A, Balsano C, Benvenuto R, Balsano F. Recognition of hepatitis B virus envelope proteins by liver-infiltrating T lymphocytes in chronic HBV infection. J Immunol 1989;143:2650-2655.

35) Nayersina R, Fowler P, Guilhot S, Missale G, Cerny A, Schlicht HJ, et al. HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. J Immunol 1993;150:4659-4671.

36) Bertoletti A, Gehring AJ. The immune response during hepatitis B virus infection. J Gen Virol 2006;87:1439-1449.

37) Lee J, Park JS, Moon JY, Kim KY, Moon HM. The influence of glycosylation on secretion, stability, and immunogenicity of recombinant HBV pre-S antigen synthesized in Saccharomyces cerevisiae. Biochem Biophys Res Commun 2003;303:427-432.

38) Shouval D, Roggendorf H, Roggendorf M. Enhanced immune response to hepatitis B vaccination through immunization with a Pre-S1/Pre-S2/S vaccine. Med Microbiol Immunol 2015;204:57-68.

39) Krawczyk A, Ludwig C, Jochum C, Fiedler M, Heinemann FM, Shouval D, et al. Induction of a robust T- and B-cell immune response in non- and low-responders to conventional vaccination against hepatitis B by using a third generation PreS/S vaccine. Vaccine 2014;32:5077-5082.

40) Madalinski K, Sylvan SP, Hellstrom U, Mikolajewicz J, Dzierzanowska-Fangrat K. Presence of anti-preS1, anti-preS2, and anti-HBs antibodies in newborns immunized with Bio-Hep-B vaccine. Med Sci Monit 2004;10:PI10-17.

41) Rendi-Wagner P, Shouval D, Genton B, Lurie Y, Rumke H, Boland G, et al. Comparative immunogenicity of a PreS/S hepatitis B vaccine in non- and low responders to conventional vaccine. Vaccine 2006;24:2781-2789.

42) Schumann A, Fiedler M, Dahmen U, Grosse-Wilde H, Roggendorf M, Lindemann M. Cellular and humoral immune response to a third generation hepatitis B vaccine. J Viral Hepat 2007;14:592-598.

43) Sylvan SP, Madalinski K, Hellstrom UB. Anti-preS responses influence the anti-HBs response in newborns after vaccination with the third generation Sci-B-Vac vaccine. Vaccine 2009;28:446-451.

44) Raz R, Dagan R, Gallil A, Brill G, Kassis I, Koren R. Safety and immunogenicity of a novel mammalian cell-derived recombinant hepatitis B vaccine containing Pre-S1 and Pre-S2 antigens in children. Vaccine 1996;14:207-211.

45) Shapira MY, Zeira E, Adler R, Shouval D. Rapid seroprotection against hepatitis B following the first dose of a Pre-S1/Pre-S2/S vaccine. J Hepatol 2001;34:123-127.

46) Pennock ND, Kedl JD, Kedl RM. T Cell Vaccinology: Beyond the Reflection of Infectious Responses. Trends Immunol 2016;37:170-180.

47) Reynolds TD, Buonocore L, Rose NF, Rose JK, Robek MD. Virus-Like Vesicle-Based Therapeutic Vaccine Vectors for Chronic Hepatitis B Virus Infection. J Virol 2015;89:10407-10415.

48) King TH, Kemmler CB, Guo Z, Mann D, Lu Y, Coeshott C, et al. A whole recombinant yeast-based therapeutic vaccine elicits HBV X, S and Core specific T cells in mice and activates human T cells recognizing epitopes linked to viral clearance. PLoS One 2014;9:e101904.

49) Bian Y, Zhang Z, Sun Z, Zhao J, Zhu D, Wang Y, et al. Vaccines Targeting PreS1 Domain Overcome Immune Tolerance in HBV Carrier Mice. Hepatology 2017.

50) Ferrari C, Cavalli A, Penna A, Valli A, Bertoletti A, Pedretti G, et al. Fine specificity of the human T-cell response to the hepatitis B virus preS1 antigen. Gastroenterology 1992;103:255-263.

Table 1. preS-specific T cell epitopes.

Epitope	Residues	Amino acid sequence	Reference
1	preS1 10-19	PLGFFPDHQL	(50)
2	preS1 41-56	WPAANQVGVGAFGPGL	(21)
3	preS2 109-134	MQWNSTAFHQALQDPR VRGLYLPAGG	(22)

Figure legends

Fig. 1. Construction and production of preS VLP. (A) Schematic representation of pCAGGS-preS-HA and pCAGGS-M1 used for preS VLP production, and pET28b-preS used for the expression of recombinant preS antigen. (B) qRT-PCR analysis was used to confirm the transcription of preS-HA and M1. (C, D) Immunofluorescent imaging of preS-HA expressing cells. (C) 293T cells were transfected with mock control, pCAGGS-M1, pCAGGS-preS-HA, or both plasmids. Cells were not permeabilized with Triton X-100 before staining. The nuclei were stained with DAPI (blue), and the preS antigen was stained with purified polyclonal rabbit anti-preS antibody, detected with Alexa Fluor® 488-Conjugated goat anti-rabbit secondary antibody. (D) The cells were the same as those in panel C, except that the cells were permeabilized with Triton X-100 before staining.

Fig. 2. Characterization of preS VLP. (A) SDS-PAGE analysis of fractions from sucrose gradient centrifugation. The major protein component is in the 40% sucrose fraction. The position of M1 is indicated by the arrow. (B) Western blot analysis with purified polyclonal rabbit anti-preS antibody. Lanes 1 and 2 showed the presence of the preS-HA antigen in fractions from sucrose gradient centrifugation. The unstained region corresponds to M1. (C) The sample in 40% sucrose fraction was treated with Peptide -N-Glycosidase F (PNGase F) and/or *O*-Glycosidase, and the preS antigen was detected by western blot. (D) Identification of glycosylated sites in preS-HA. The preS domain in preS-HA is colored black. Glycosylated residues identified in this study are underlined. Glycosylated residues which have

been reported previously are indicated by dots[18]. (E) An electron micrograph showing preS virus-like particles (preS VLP). Magnification, 11,000 ×.

Fig. 3. preS VLP elicits superior neutralizing antibodies compared to recombinant preS vaccination. Balb/c mice were immunized intramuscularly with preS VLP (n=6), recombinant preS protein (n=6), or PBS (n=6). (A) Time schedule for preS VLP vaccination to prevent hydrodynamic transfection of HBV DNA. (B-D) The serum anti-preS titers were determined by ELISA. The plates were coated with 1 μg/mL purified recombinant preS. The immunization condition is labeled next to chart color codes. The sera were diluted by 100 folds in all the assays. (E) Neutralization of HBV infectivity in HepG2/hNTCP cells by mouse anti-preS VLP sera or hepatitis B immunoglobulin (HBIG) (0.144 mg/mL). The mouse anti-preS VLP sera were diluted by various folds. HBeAg values at one week post infection were measured using an ELISA kit. The data are represented as mean ± SEM.

Fig. 4. preS VLP induces stronger T cell responses than recombinant preS vaccination. Balb/c mice were immunized intramuscularly with preS VLP (n=6), recombinant preS protein (n=6), or PBS (n=6). 30 days postimmunization, splenocytes were isolated and analyzed for CD8, CD4, and IFN-γ expression by flow cytometry (A-B). $CD8^+$ T cells (A) or $CD4^+$ T cells (B) were gated, and IFN-γ-producing cells are presented as the percent average from each group. (C) Splenocytes were isolated and analyzed for IFN-γ expression using an IFN-γ ELISPOT assay. ELISPOT experiments were performed in triplicate wells per condition. (D) Representative images of ELISPOT from each group are shown. All values are presented as the average from each group, and error bars indicate ± SEM. Pool, indicates the mixture of peptide 10, 41 and 109, and preS indicates recombinant preS protein.

Fig. 5. Immunization with preS VLP offers protection against hydrodynamic transfection of HBV DNA. Balb/c mice were immunized intramuscularly with preS VLP (n=6), recombinant preS protein (n=6), or PBS (n=6). On day 70, HBV replication was induced by hydrodynamic injection of pT-HBV1.3 plasmid via tail vein (10 μg per mouse). (A) Liver-associated HBV RNA copies were measured by qRT-PCR. (B) Immunohistochemistry analysis of liver tissues. Quantifications represent average numbers of HBcAg-positive cells per 100× field (n = 3 with 5 fields counted per sample). Representative images are shown. Magnification, 100×. Serum analysis for HBsAg (C), HBeAg (D) and anti-preS antibody titers (E) was

completed by ELISA on days 0, 2, 4, and 7 post transfection. (F) Serum alanine aminotransferase (ALT) activity was determined with a Hitachi 7600 Automatic Biochemistry Analyzer. All values are presented as the average from each group, and error bars indicate ± SEM.

Fig. 6. Immunization with preS VLP induces robust anti-preS antibodies and T cell responses in HBV transgenic mice. HBV transgenic mice were immunized intramuscularly with preS VLP (n=6) or PBS (n=6). (A) Time schedule for preS VLP as a vaccine to treat HBV transgenic mice. (B-D) The serum anti-preS titers were determined by ELISA. The plates were coated with 1 μg/mL purified recombinant preS. The immunization condition is labeled next to chart color codes. The sera were diluted by 100 folds in all the assays. $CD8^+$ T cells (E) or $CD4^+$ T cells (F) were gated, and IFN-γ-producing cells are presented as the percent average from each group. (G) On day 70, splenocytes were isolated and analyzed for IFN-γ expression by ELISPOT assays. ELISPOT experiments were performed in triplicate wells per condition. (H) Representative images of ELISPOT from each group are shown. All values are presented as the average from each group, and error bars indicate ± SEM.

Supporting Information

A Virus-like Particle of the Hepatitis B Virus preS Antigen Elicits Robust Neutralizing Antibody and T Cell Responses in Mice

Xiaodan Cai [a,†], Weihao Zheng [a,†], Shaokun Pan [b], Shengyuan Zhang [c], Youhua Xie [b], Haitao Guo [d], Guoxin Wang [e,*], Zigang Li [a,*], and Ming Luo [f,g,*]

Supplementary Materials and Methods

Plasmids and cells

The vector for expressing HBV preS (adw subtype, Accession Number AGW20902) in *E. coli* (pET28b-preS) was constructed previously [(5)]. The His-tagged preS protein was expressed and purified as described previously [(5)]. Recombinant preS was quantitated by the bicinchoninic acid assay (BCA assay). To prepared polyclonal rabbit anti-preS antibody, purified recombinant preS protein was mixed with Alum adjuvant and injected subcutaneously into New Zealand rabbits four times in two months, and the antisera was collected from the rabbits two weeks postimmunization and was subjected to affinity chromatography. The plasmids for expressing M1 protein (the matrix protein) of influenza virus A/sw/Spain/53207/04 and a preS-HA (HA=hemagglutinin) chimeric protein were constructed by inserting the coding sequence in pCAGGS. The amino acid 41 of M1 was mutated to Ala (pCAGGS-M1). The preS-HA has the sequence of HBV preS followed by aa521-566 of HA (pCAGGS-preS-HA). 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen). All the fetal bovine serum used in this study was inactivated at 56°C for 30 min.

Preparation and characterization of the virus-like particles

The pCAGGS-M1 and pCAGGS-preS-HA plasmids were transfected into 293T cells with polyethylenimine. 72 h after transfection, the culture medium was centrifuged at 6,000 rpm for 15 min at 4°C to remove cellular debris, followed by centrifugation at 22,000 rpm for 3 h at 4°C. The pellet was resuspended in PBS at 4°C overnight, and further purified through a 20%-60% sucrose gradient in a Beckman SW41Ti rotor at 30,000 rpm for 3 h at 4°C. The 40% sucrose fraction was harvested and diluted with PBS by about 5 folds. After centrifugation at 22,000 rpm for 3 h at 4°C to remove the sucrose, the virus-like particles were resuspended in PBS at 4°C overnight. Quantitation of preS VLP total proteins was carried out by the bicinchoninic acid assay (BCA assay). A sample was applied to a 400mesh carbon-coated copper grid, and stained with 1% phosphotungstic acid (J&K Scientific). preS VLP was visualized on a Tecnai G^2 Spirit transmission election microscope operating at 120 kV.

Indirect immunofluorescence

293T cells were grown on glass coverslips and transfected with pCAGGS-M1 and pCAGGS-preS-HA. 48 h posttransfection, cells were fixed with 4% paraformaldehyde. Cells were classified into two groups. One was permeabilized with 0.2% Triton X-100 for 5 min, the other without permeabilization. After blocking for 1 h in PBS containing 5% goat serum, all cells were incubated with purified polyclonal rabbit anti-preS antibody at 4°C overnight. Cells were washed with PBS following incubation with Alexa Fluor® 488-Conjugated goat anti-rabbit secondary antibody (ZSGB-BIO, Beijing, China) for 1 h at 37°C. After washing, cells were stained with DAPI for 10 min, and then mounted onto microscope slides. Confocal slices were acquired with a 100× objective, using a Zeiss 510 confocal microscope with random sampling.

Western blot analysis

Western blot analysis was performed as described previously [(6)]. Briefly, samples were mixed with 4 × SDS-PAGE loading buffer followed by heating at 95°C for 10 min. The denatured samples were separated by electrophoresis on 12%-SDS-PAGE gel and transferred to a PVDF membrane. After blocking with a 5 % (wt/vol) skim milk solution, the membrane was incubated with purified polyclonal rabbit anti-preS antibody (1:1,000) and then with HRP-conjugated goat anti-rabbit IgG antibody (Abclonal). The chemiluminescent detection reaction was performed and monitored by ChemiDoc XRS System (Bio-Rad).

Glycosylation analysis

The glycosylation of preS-HA in VLP was analysed by western blot and LC-MS/MS. For western blot analysis, the sample from 40% sucrose fraction was treated with PNGase F and/or *O*-Glycosidase (New England Biolabs) following the manufacturer's instructions. Briefly, 2 μL of samples, 1 μL of Glycoprotein Denaturing Buffer (10×) and 7 μL of H_2O were combined in a total reaction volume of 10 μL, and samples were denatured by heating the reaction mixture at 100°C for 10 min followed by cooling to room temperature. 2 μL of GlycoBuffer 2 (10×) and 2 μL of 10% NP-40, 2 μL of Neuraminidase (only used for *O*-Glycosidase), H_2O, and 1 μL of *O*-Glycosidase and/or PNGase F were combined in a total reaction volume of 20 μL, followed by incubation at 37°C for 18 h. Samples were subsequently subjected to western blot analysis as described above.

For LC-MS/MS analysis, the sample from 40% sucrose fraction was subjected to electrophoresis on a 12%-SDS-PAGE gel, which was stained by coomassie R250. The bands corresponding to 25-30 kDa were cut out, subjected to reduction with 10 mM DTT at 56°C for 50 min, and subsequent alkylated with 55 mM iodoacetamide (IAA) for 30 min at room temperature in the dark. In-gel digestion was conducted with

trypsin [Promega, enzyme: protein= 1:50 (wt/wt)] at 37°C for 16 h in 25 mM ammonium bicarbonate buffer, followed by lyophilization.

The lyophilized samples were re-dissolved in 2% acetonitrile, 0.1% formic acid, and loaded on a ChromXP C18 (3 μm, 120 Å) nanoLC trap column. The inline trapping, desalting procedure was carried out at a flow rate of 2 μL/min for 10 min with 100% solvent A (Solvent A: water/acetonitrile/formic acid = 98/2/0.1% solvent B: 2/98/0.1%). A 60-min gradient elution ranging from 5-35% acetonitrile (0.1% formic acid) was carried out onto an analytical column (75 μm x 15 cm C18- 3μm 120 Å, ChromXP Eksigent). LC-MS/MS analysis was performed with a Triple TOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON). Data were acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 PSI, nebulizer gas of 5 PSI, and an interface heater temperature of 150 °C. The MS instrument was operated as TOF-MS scans. For IDA, survey scans were acquired in 250 ms and as many as 25 product ion scans (90 ms) were collected if exceeding a threshold of 150 counts per second (counts/s) and with a +2 to +4 charge-state. A Rolling collision energy setting was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for ½ of peak width (~12 s).

For glycosylation analysis, the raw files were searched against the local database including the protein sequences for M1, preS-HA and decoys, using Byonic (version 2.11.0). All of the N-glycan and O-glycan databases were used for searching possible glycan modifications, with the following parameters: mass tolerances were 10 ppm and 40 ppm for the precursor and fragment ions, respectively; enzyme specificity was set as RK, with a maximum of two missed cleavages; carbamidomethylation of Cys residues were set as fixed modification (57.021464 Da); variable modifications including oxidation of Met residues, acetylation of protein N-terminals, and carbamidomethylation of N-terminal, His and Lys were allowed; the 1% False Discovery Rate Analysis was also engaged. Glycopeptides were filtered using the following criteria: Score≥300, Delta Mod≥10, |Log Probability|≥2.

Immunization and hydrodynamic transfection in Balb/c mice

All mouse experiments were conducted in accordance with the institutional guidelines following the experimental protocol reviewed and approved by the Institutional Animal Care and Use Committee of Peking University. Female Balb/c mice of 6-8 weeks old were immunized by injecting the antigen preparation in the hindlimb. A booster was given on day 22. Blood was collected on day 52 and 112, and neutralizing antibody titers were determined by ELISA. On day 52, activated T cells in splenocytes were analyzed by ELISPOT and flow cytometry (FACS). The immunized mice were hydrodynamically transfected on day 70. 10 μg of pHBV1.3, a plasmid containing 1.3X genome length of HBV, genotype D, ayw subtype, was used in hydrodynamic injection to establish HBV replication as previously described [7]. Blood samples were

collected at different time points to measure HBV antigens. On day 77, mice were sacrificed and liver tissues were used for measuring antigens and RNA of HBV.

HBV infection and neutralization assays

Virus neutralization assays were conducted as described previously [8, 9]. HepAD38 cells were cultured in Dulbecco's modified Eagle's medium F12 (DMEM F12) (Invitrogen, Carlsbad, USA) containing 2 mM L-glutamine, 50 U/ml penicillin, and 10% fetal bovine serum (Invitrogen) at 37°C and 5% CO2. The virus-containing supernatant from HepAD38 culture was concentrated using Amicon Ultra-15 centrifugal filters (Millipore, Billerica, USA). HBV titers were quantified using a qRT-PCR HBV DNA detection kit (Qiagen, Hilden, Germany) and expressed as genome equivalents (geq). For infection experiments, 5×10^5 HepG2/hNTCP cells per well were cultured in 24-well plate and incubated overnight with the viral inoculum (M.O.I. of 500) alone or together with various dilutions of mouse anti-preS VLP sera or 1,000 fold dilution of hepatitis B immunoglobulin (HBIG) (from Chengdu Rongsheng Bioproduct Company, with a protein concentration of 144 mg/ml), with 4% PEG present during virus infection. Medium was changed every 2 or 3 days, and HBeAg was measured at 1 week post infection using Diagnostic ELISA Kit for Hepatitis B e antigen (Kehua Bio-engineering).

Isolation of splenocytes

For splenocyte isolation, splenocytes were gently grinded followed by passaging through 40 μm strainers and treating with ACK lysing buffer. After washing with PBS, cells were resuspended in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% Penicillin-Streptomycin–L-Glutamine.

Enzyme-linked immunospot assay

T cell responses were determined using an IFN-γ ELISPOT set (BD Biosciences) following the manufacturer's protocol. Briefly, 96-well plates were coated with purified anti-mouse IFN-γ antibody (1:200) at 4°C overnight, and then were blocked for 2 h using DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% Penicillin-Streptomycin– L-Glutamine. Splenocytes were seeded at 2×10^5/well. Peptides representing previously described epitopes present in preS (Table 1) or purified recombinant preS protein at a concentration of 20 μg/ml were used to stimulate cells for 36 h at 37°C in a 5% CO_2 and humidified incubator, with media and phorbol myristate acetate (PMA)/ionomycin-treated cells used as negative and positive controls, respectively. After being washed, cells were incubated with biotinylated anti-mouse IFN-γ antibody (1:250) for 2 h at room temperature, and then incubated with streptavidin-horseradish peroxidase (HRP) (1:1,000) for 1 h. Following the final washes, 3-amino-9-ethylcarbazole (AEC) substrate (Alfa Aesar) was added to the wells and allowed to develop at room temperature for 25 to 35 min. The reaction

was stopped with distilled water, and the plates were allowed to air dry before spot-forming cells were enumerated automatically with an ELISPOT plate reader.

Flow cytometry

Splenocytes were resuspended in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% Penicillin-Streptomycin– L-Glutamine, and then were seeded at 2×10^6 /well. The cells were then stimulated for 6 h with preS-specific peptides or purified recombinant preS protein diluted to a final concentration of 10 μg/ml in DMEM supplemented with 2 μg/ml brefeldin A (BD Biosciences). The cells were then washed in staining buffer (PBS containing 2% fetal bovine serum) and stained for CD8 and CD4 surface expression for 30 min at 4°C using fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8 antibody (BD Biosciences) and peridinin chlorophyII protein(PerCP)-conjugated anti-mouse CD4 antibody (BD Biosciences). Then the cells were washed, fixed, and permeabilized using a commercially available Cytofix/Cytoperm kit (BD Biosciences). The cells were then stained for 30 min at 4°C for intracellular cytokine expression using phycoerythrin (PE)-conjugated anti-mouse IFN-γ antibody (BD Biosciences). After washing, cells were resuspended in staining buffer and analysed using a BD FACS Canto™ II flow cytometer (BD Biosciences) and FACSDiva Version 6.1.3. Results were generated from data gathered from 200,000 cells.

ELISA

Purified recombinant preS antigen (1 μg/ml) was absorbed to 96 well plates, blocked with 10% BSA, and then 50 μl of 1:100 dilution of sera was added and incubated for 30 min at 37°C. The plates were washed six times, and followed by incubation with a 1:5,000 dilution of HRP-conjugated anti-mouse IgG, IgG1 (Santa Cruz Biotechnology) or IgG2a (BD Biosciences) for 30 min at 37°C. After washing, the plates were incubated with TMB substrate for 10 minutes before stopping with 2 M H_2SO_4. Absorbance at 450 nm was measured by the 2104 EnVision® Multilabel Reader (PerkinElmer). Endpoint dilutions were performed (Fig. S5). In addition, serum samples were diluted 1:5 for HBsAg ang HBeAg detection (Kehua Bio-engineering).

ALT activity analysis

The level of serum alanine aminotransferase (ALT) was measured with a Hitachi 7600 Automatic Biochemistry Analyzer.

RT-PCR and qRT-PCR analyses

For detecting mRNA in 293T cells transfected with expression vectors, 48 h posttransfection, total RNAs from 293T cells were extracted using the QIAGEN RNeasy Mini Kit following the manufacturer's instructions. Total RNAs were stored at -20°C until being used. Any possible contaminating DNA was digested by DNase I

(Takara). Total RNAs were quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific). 1 μg total RNAs were reverse transcribed into cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara) in a 20 μL reaction as described previously [10]. cDNA derived from 5 ng total RNAs was used as template for qRT-PCR amplification. Primers (preS-HA-FW: 5'-CCACCAATCGGCAGTC-3') and (preS-HA-RV: 5'-GCCACCAGCAGGAAGAT-3') were used for preS-HA transcripts; (M1-FW: 5'-TGACAACAACCAACCCACT-3') and (M1-RV: 5'-CTGCTGCTTGCTCACTCG-3') were for M1 transcripts; (β-actin-FW: 5'-TCATGAAGTGTGACGTGGACATC-3') and (β-actin-RV: 5'-CAGGAGGAGCAATGATCTTGATCT-3') were used for β-actin transcripts. qRT-PCR was performed with GoTaq qPCR Master Mix (Promega) following the manufacturer's protocol for a total reaction volume of 20 μL in the CFX96 Real-Time PCR Detection System (Bio-Rad). The reaction product was subjected to agarose gel electrophoresis.

For hydrodynamic transfection studies, total RNAs from the liver of HBV-transfected mice were isolated with Trizol reagent (Invitrogen) according to manufacturer's manual. Any possible contaminating DNA was digested by DNase I (Takara). Total RNAs were quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific). 1 μg total RNAs were reverse transcribed into cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara) in a 20 μL reaction as previously described[10]. cDNA derived from 2 ng total RNAs was used as template for qRT-PCR amplification. Primers (HBV2270FW: 5'-GAGTGTGGATTCGCACTCC-3') and (HBV2392RV: 5'-GAGGCGAGGGAGTTCTTCT-3') were used for HBV RNA transcripts [2]. qRT-PCR was conducted with GoTaq qPCR Master Mix (Promega) following the manufacturer's instructions for a total reaction volume of 20 μL in the CFX96 Real-Time PCR Detection System (Bio-Rad).

H&E staining and immunohistochemistry

Liver tissues were collected and fixed in 10% neutral formalin (Sigma). After paraffin embedding, liver sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed using polyclonal rabbit anti-HBcAg antibody (Dako) to detect HBV core antigen.

Immunization in HBV transgenic mice

All mouse experiments were performed in accordance with the institutional guidelines following the experimental protocol reviewed and approved by the Institutional Animal Care and Use Committee of Peking University. Six- to 8-week-old female HBV transgenic mice (ayw subtype) were obtained from Infectious Disease Center of No. 458 Hospital (Guangzhou, China). HBV transgenic mice were immunized intramuscularly with 20 μg of preS VLP in the hindlimb, and were boosted on days 22 and 43, respectively. Blood was collected on days 0, 30 and 70, and anti-preS

antibody titers were determined by ELISA. On day 70, activated T cells in splenocytes were analyzed by ELISPOT and flow cytometry.

Statistical analysis

The data are represented as mean ± SEM. The statistical difference was determined by a two-tailed Student's t-test (*P<0.05; **P<0.01; ***P<0.001 vs. the mock group).

Table S1: Identification of glycosylated residues of preS-HA.

Peptide sequence	Glycans	|Log Prob|	Score	Delta Mod. Score
R.DSHPQAMQWN[+1257.44941]STAFHQALQDPR.V	HexNAc(3)Hex(4)	4.15	343	235.9
R.QPTPIS[+406.15875]PPLR.D	HexNAc(2)	6.66	528.6	92.2
R.QPTPIS[+203.07937]PPLR.D	HexNAc(1)	8.14	617	45.1
R.QPTPIS[+963.31794]PPLR.D	HexNAc(1)Hex(1)NeuGc(1)NeuAc(1)	4.98	354	39.3
R.QPTPIS[+730.26439]PPLR.D	HexNAc(2)Hex(2)	5.32	440.1	38.1
R.QPTPIS[+947.32303]PPLR.D	HexNAc(1)Hex(1)NeuAc(2)	7.32	514.1	38.1
R.QPTPIS[+568.21157]PPLR.D	HexNAc(2)Hex(1)	6.65	572.1	33.8
R.QPTPIS[+365.13220]PPLR.D	HexNAc(1)Hex(1)	8.14	619.8	32.4
R.QPTPIS[+656.22761]PPLR.D	HexNAc(1)Hex(1)NeuAc(1)	8.55	603.2	31.7
R.QPTPIS[+859.30699]PPLR.D	HexNAc(2)Hex(1)NeuAc(1)	4.43	421.9	24.1
R.QSGRQPTPIS[+656.22761]PPLR.D	HexNAc(1)Hex(1)NeuAc(1)	5.12	466.8	17.2

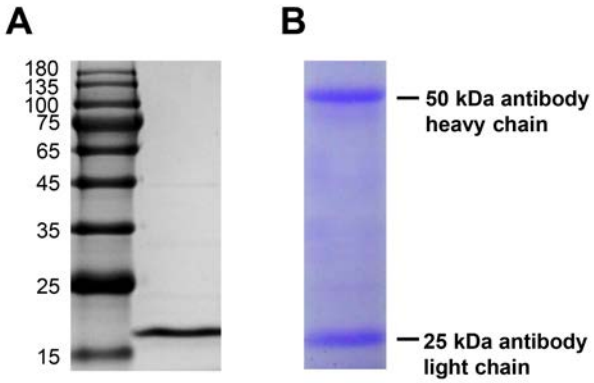


Fig.S1: SDS-PAGE analysis of purified recombinant preS protein (A) and polyclonal rabbit anti-preS antibody (B).

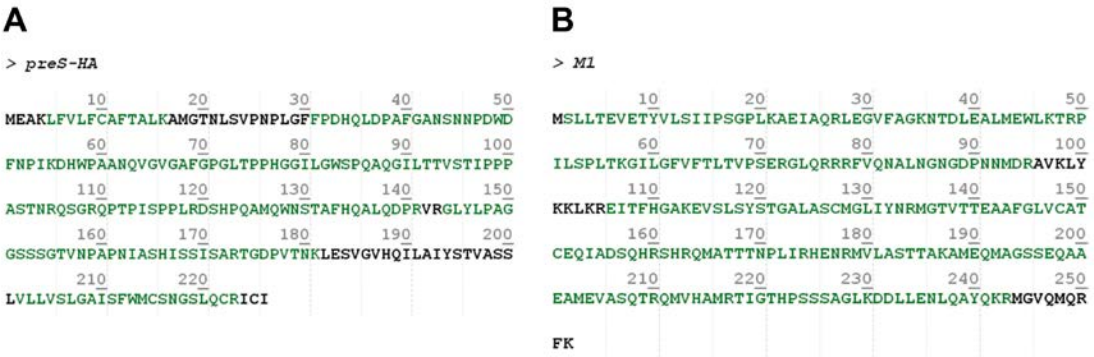


Fig.S2: LC-MS/MS identification of M1 and preS-HA in the sample from 40% sucrose fraction. The identified sequences are colored green.

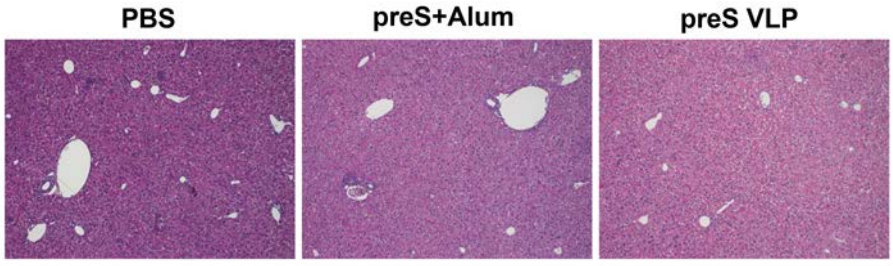


Fig.S3: Liver tissue sections of Balb/c mice from each group were stained with hematoxylin and eosin on day 7 after hydrodynamic transfection of HBV DNA. Magnification, 100×.

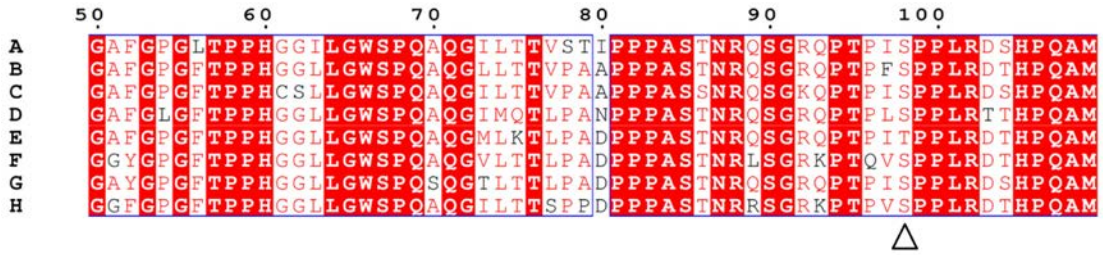


Fig.S4: Partial alignment of the primary sequences of preS from all HBV genotypes. preS in VLP is genotype A.

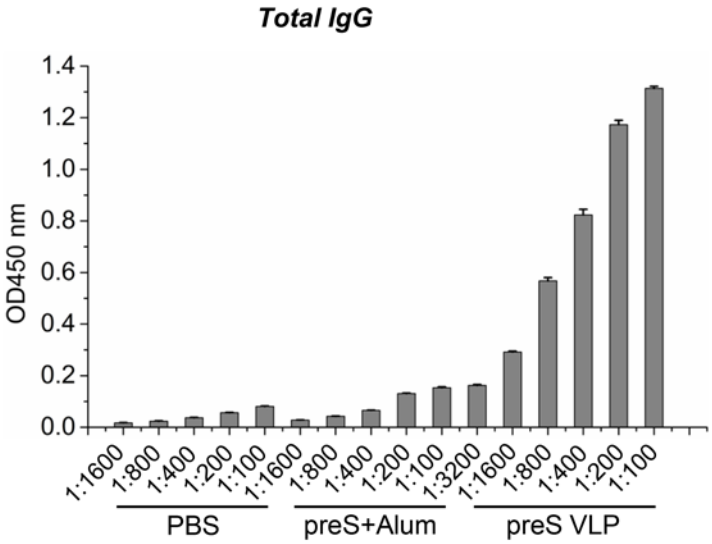
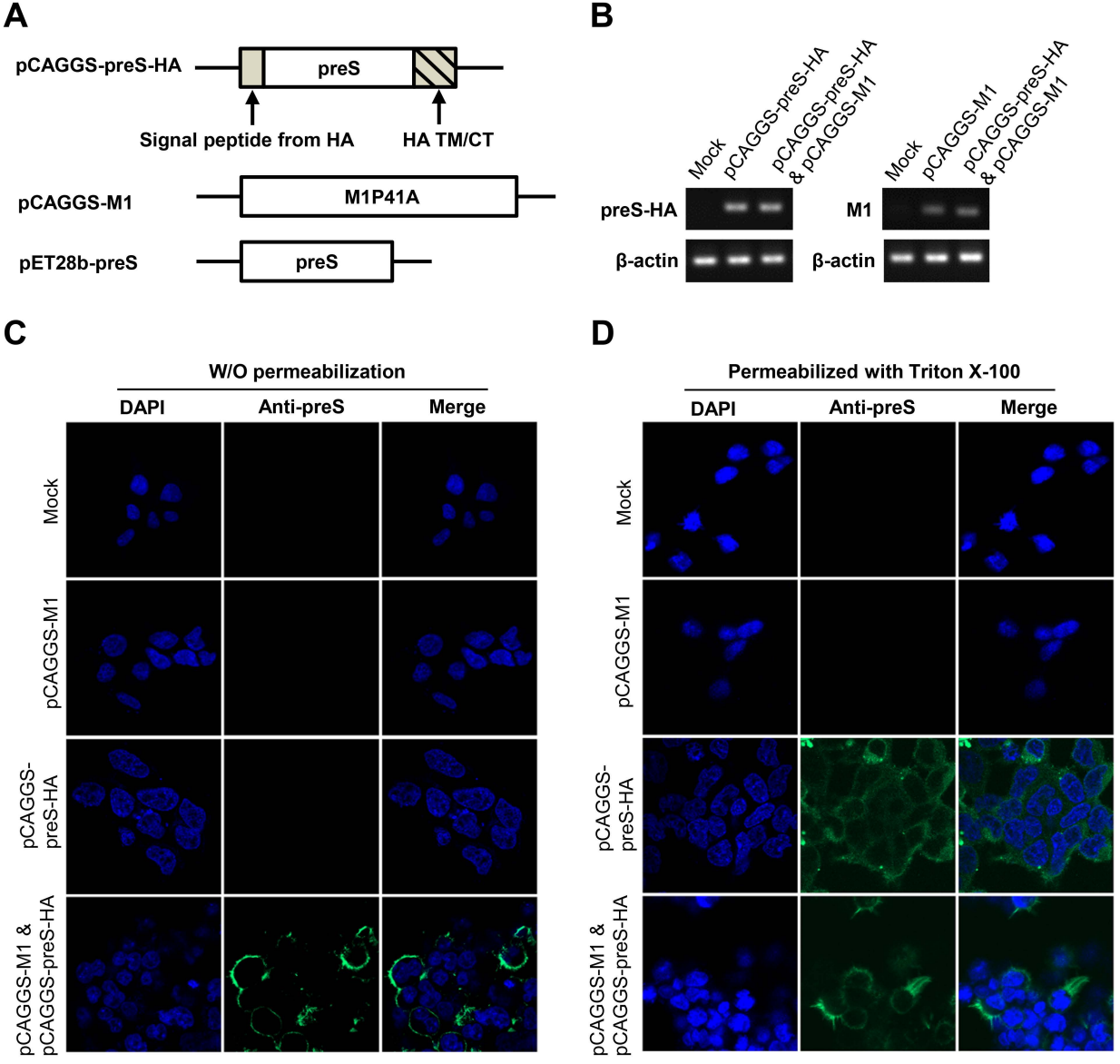
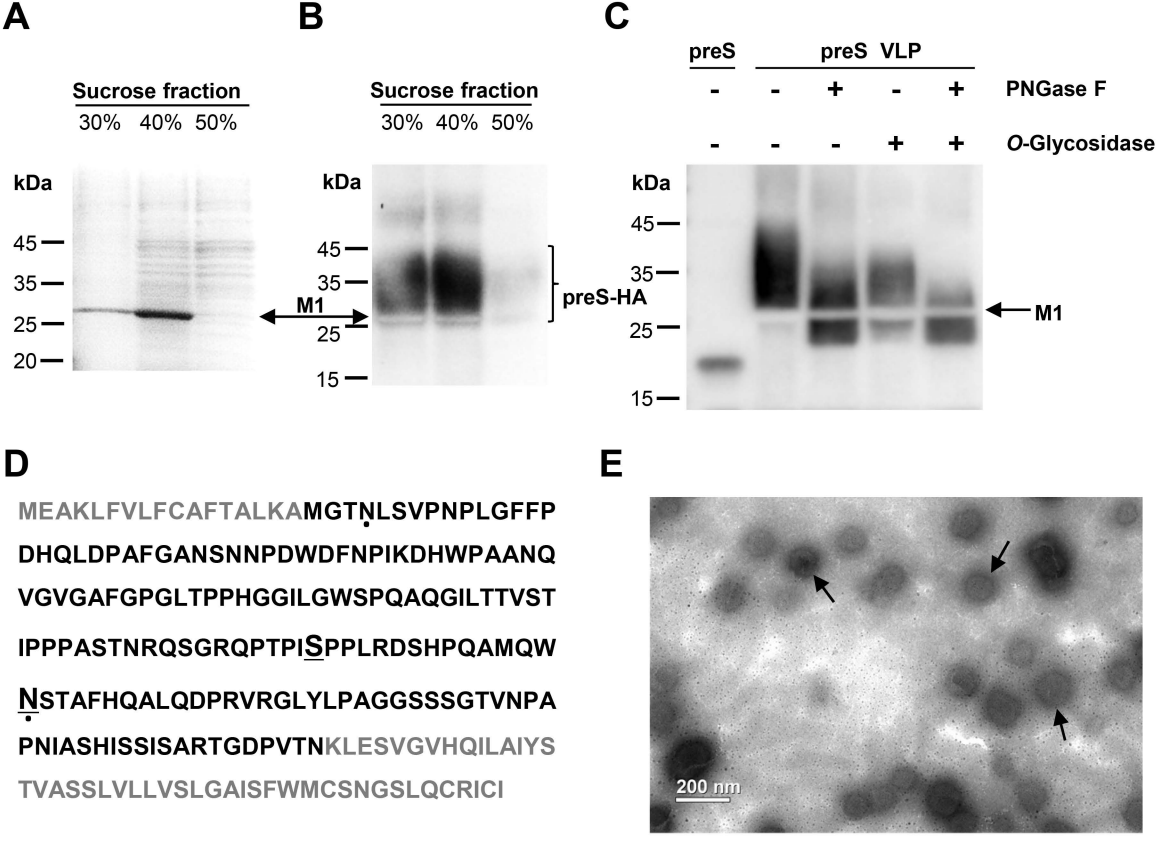
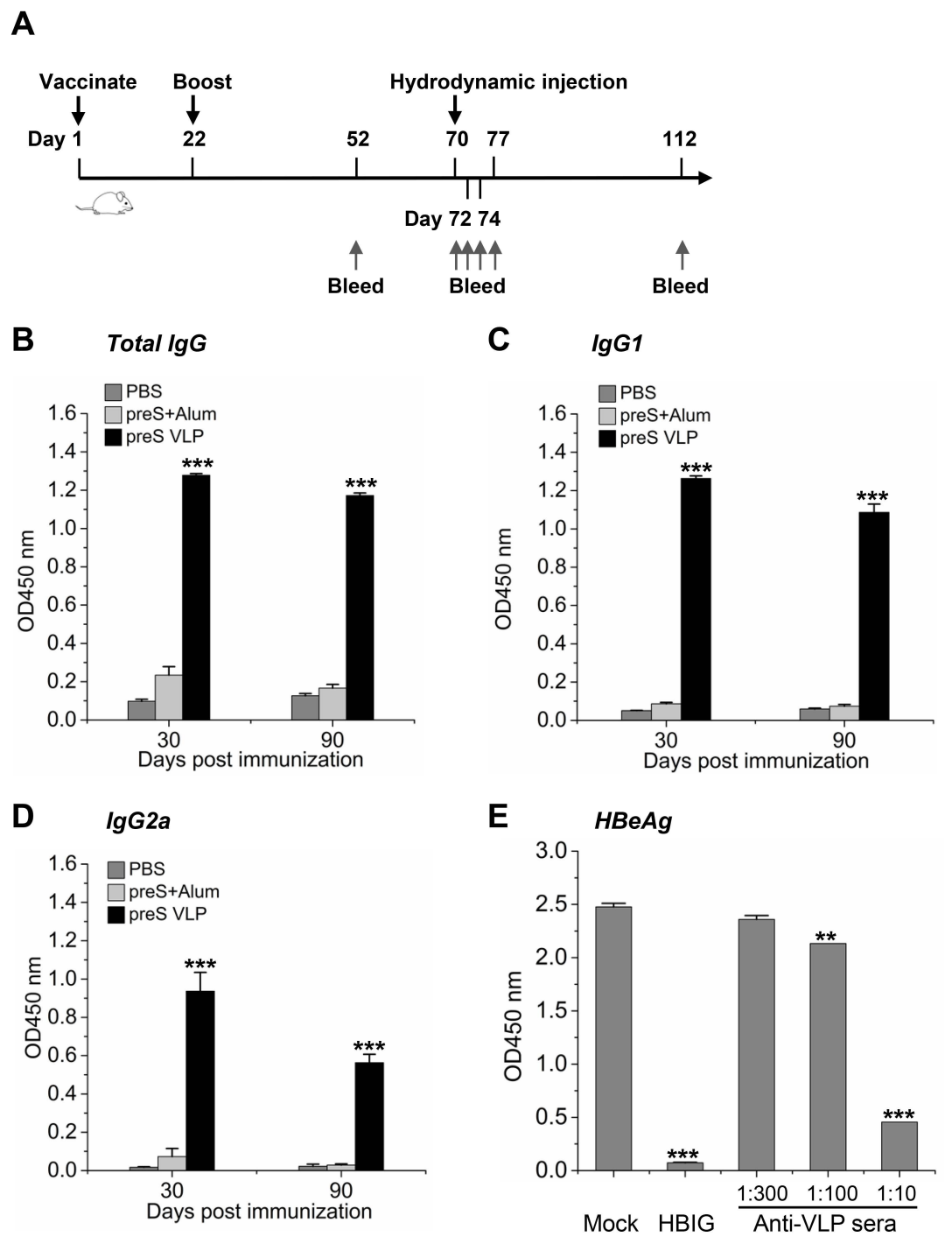
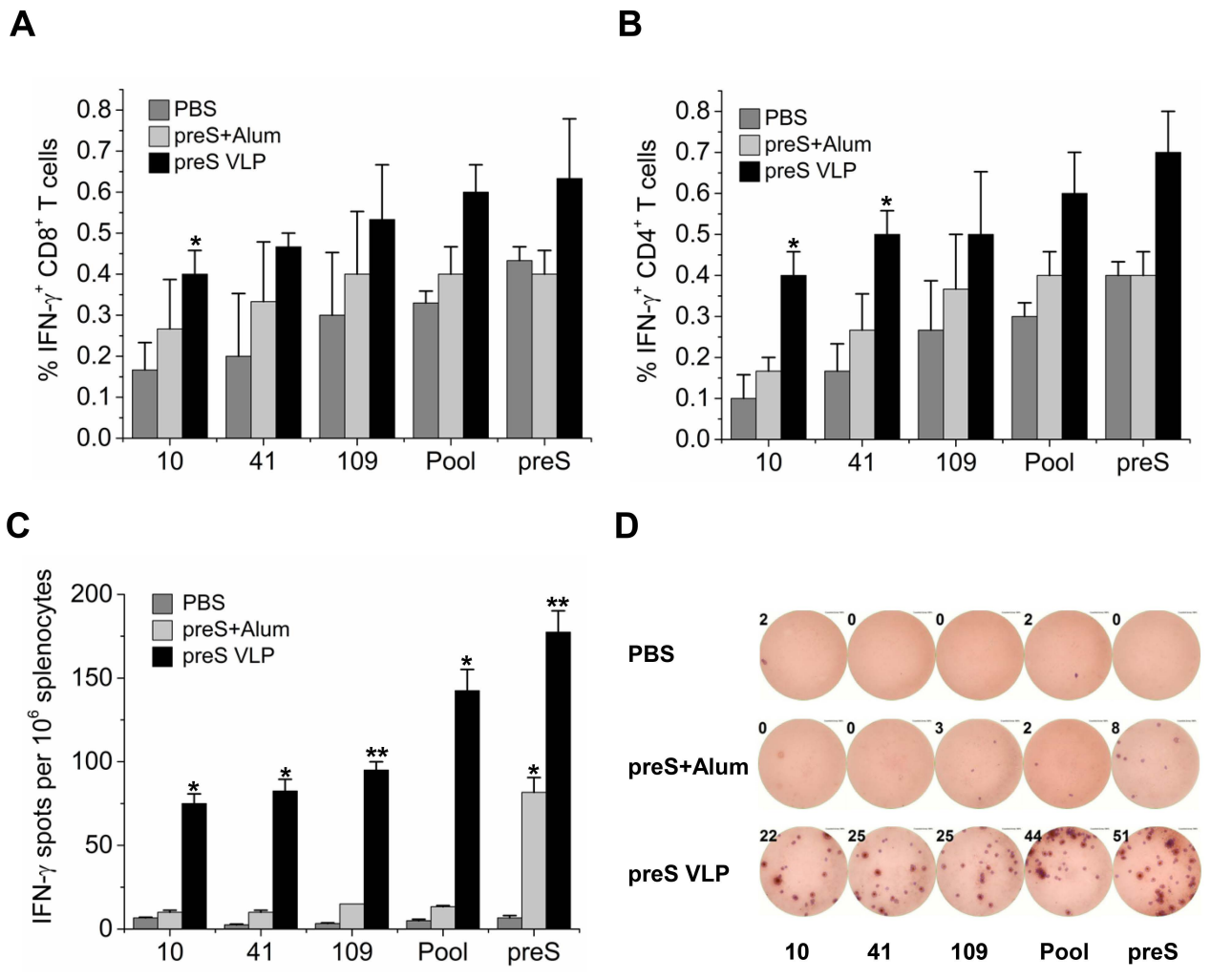


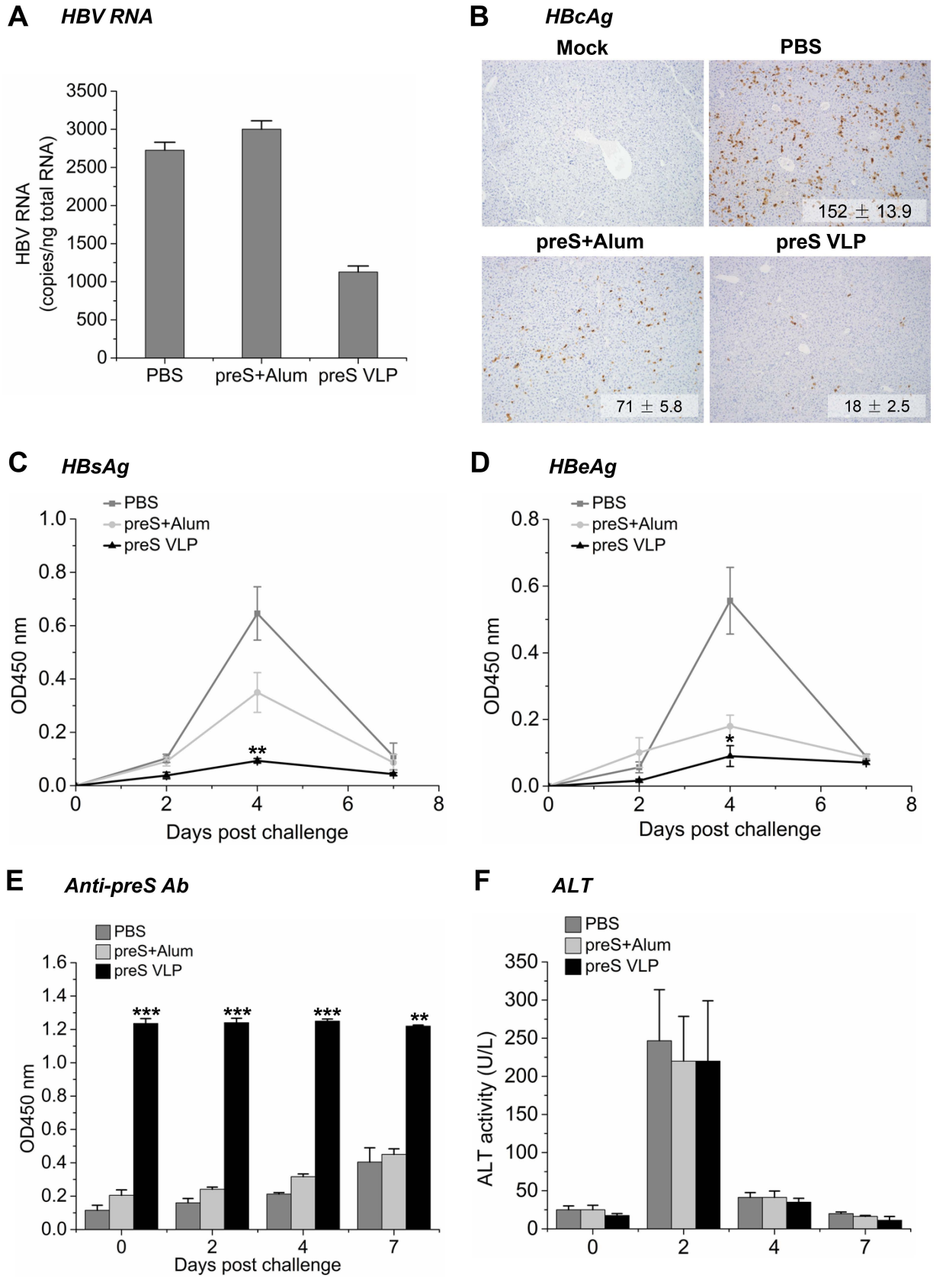
Fig.S5: Measurement of anti-preS antibodies by endpoint dilution assay. The serum anti-preS titers were determined by ELISA. The plates were coated with 1 μg/mL purified recombinant preS. The sera were diluted by various folds.

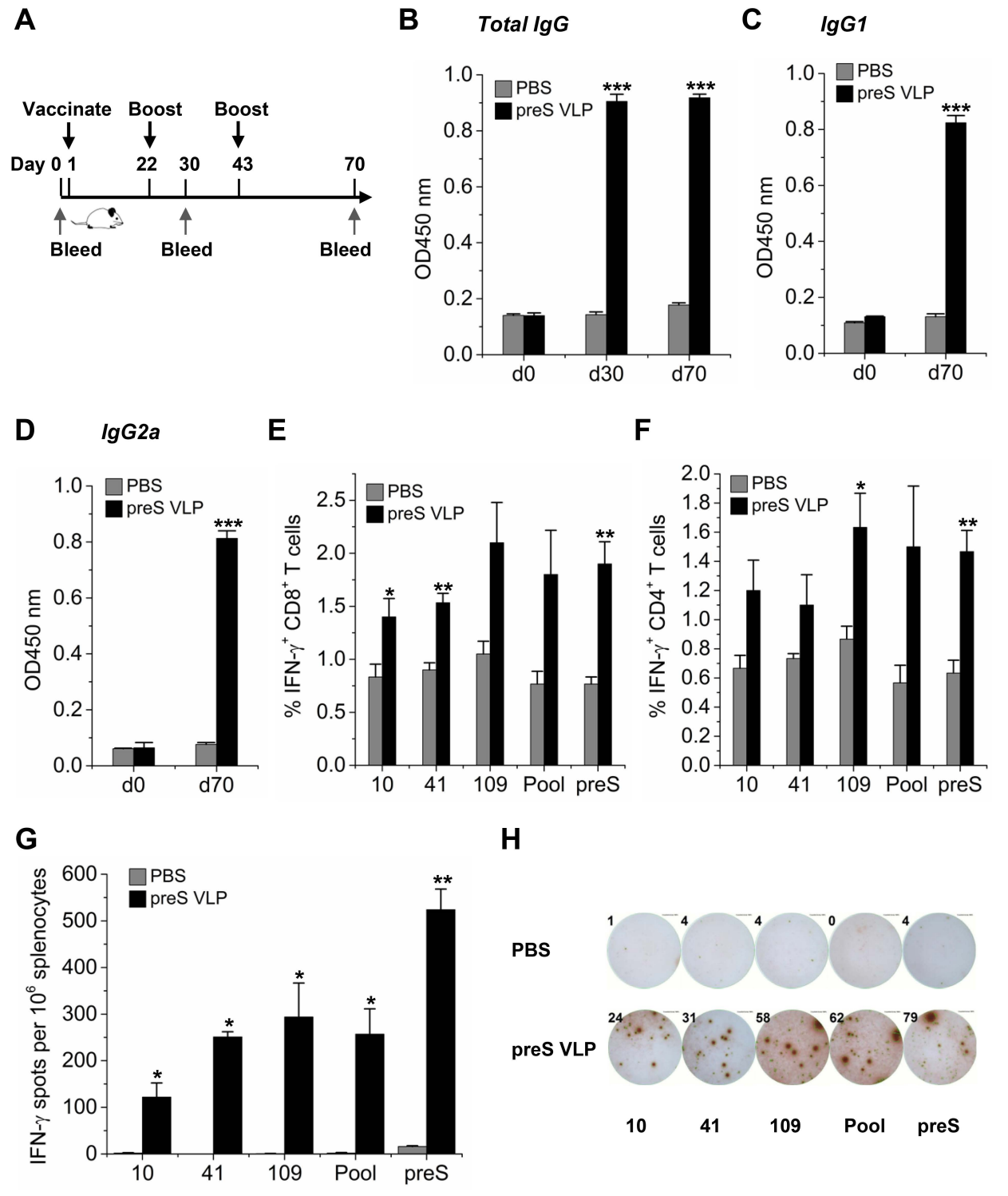

A
pCAGGS-preS-HA
preS
Signal peptide from HA
HA TM/CT
pCAGGS-M1
M1P41A
pET28b-preS
preS
B
Mock
pCAGGS-preS-HA
pCAGGS-preS-HA & pCAGGS-M1
Mock
pCAGGS-M1
pCAGGS-preS-HA & pCAGGS-M1
preS-HA
β-actin
M1
β-actin
C
W/O permeabilization
DAPI
Anti-preS
Merge
Mock
pCAGGS-M1
pCAGGS-preS-HA
pCAGGS-M1 & pCAGGS-preS-HA
D
Permeabilized with Triton X-100
DAPI
Anti-preS
Merge
Mock
pCAGGS-M1
pCAGGS-preS-HA
pCAGGS-M1 & pCAGGS-preS-HA


A
Sucrose fraction
30% 40% 50%
kDa
45
35
25
20
B
Sucrose fraction
30% 40% 50%
kDa
45
35
M1
25
15
preS-HA
C
preS
preS VLP
- - + - + PNGase F
- - - + + O-Glycosidase
kDa
45
35
25
15
M1
D
MEAKLFVLFCAFTALKAMGTNLSVPNPLGFFP
DHQLDPAFGANSNNPDWDFNPIKDHWPAANQ
VGVGAFGPGLTPPHGGILGWSPQAQGILTTVST
IPPPASTNRQSGRQPTPISPPLRDSHPQAMQW
NSTAFHQALQDPRVRGLYLPAGGSSSGTVNPA
PNIASHISSISARTGDPVTNKLESVGVHQILAIYS
TVASSLVLLVSLGAISFWMCSNGSLQCRICI
E
200 nm


A
Vaccinate
Boost
Hydrodynamic injection
Day 1
22
52
70
77
112
Day 72 74
Bleed
Bleed
Bleed
B
Total IgG
PBS
preS+Alum
preS VLP
OD450 nm
Days post immunization
C
IgG1
D
IgG2a
E
HBeAg
Mock
HBIG
1:300
1:100
1:10
Anti-VLP sera


A
% IFN-γ+ CD8+ T cells
PBS
preS+Alum
preS VLP
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
*
10
41
109
Pool
preS
B
% IFN-γ+ CD4+ T cells
PBS
preS+Alum
preS VLP
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
*
*
10
41
109
Pool
preS
C
IFN-γ spots per 10^6 splenocytes
PBS
preS+Alum
preS VLP
0
50
100
150
200
*
*
**
*
*
**
10
41
109
Pool
preS
D
PBS
preS+Alum
preS VLP
2
0
0
2
0
0
0
3
2
8
22
25
25
44
51
10
41
109
Pool
preS


A
HBV RNA
HBV RNA (copies/ng total RNA)
3500
3000
2500
2000
1500
1000
500
0
PBS
preS+Alum
preS VLP
B
HBcAg
Mock
PBS
152 ± 13.9
preS+Alum
preS VLP
71 ± 5.8
18 ± 2.5
C
HBsAg
PBS
preS+Alum
preS VLP
OD450 nm
1.0
0.8
0.6
0.4
0.2
0.0
**
0
2
4
6
8
Days post challenge
D
HBeAg
PBS
preS+Alum
preS VLP
OD450 nm
0.8
0.6
0.4
0.2
0.0
*
0
2
4
6
8
Days post challenge
E
Anti-preS Ab
PBS
preS+Alum
preS VLP
OD450 nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
**
0
2
4
7
Days post challenge
F
ALT
PBS
preS+Alum
preS VLP
ALT activity (U/L)
350
300
250
200
150
100
50
0
0
2
4
7


A
Vaccinate
Boost
Boost
Day 0 1
22
30
43
70
Bleed
Bleed
Bleed
B
Total IgG
PBS
preS VLP
OD450 nm
d0
d30
d70
C
IgG1
PBS
preS VLP
OD450 nm
d0
d70
D
IgG2a
PBS
preS VLP
OD450 nm
d0
d70
E
PBS
preS VLP
% IFN-γ+ CD8+ T cells
10
41
109
Pool
preS
F
PBS
preS VLP
% IFN-γ+ CD4+ T cells
10
41
109
Pool
preS
G
PBS
preS VLP
IFN-γ spots per 10^6 splenocytes
10
41
109
Pool
preS
H
PBS
preS VLP
1
4
4
0
4
24
31
58
62
79
10
41
109
Pool
preS